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ประวัติผู้วิจัย

1. ผ.ศ. วิมล ขวัญเกื้อ ตำแหน่ง ผู้ช่วยศาสตราจารย์ ระดับ 8

วุฒิการศึกษา กศบ. (ชีววิทยา) วิทยาลัยวิชาการศึกษาประสานมิตร

วทม. (พันธุศาสตร์) มหาวิทยาลัยเกษตรศาสตร์

ปัจจุบัน อาจารย์ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศิลปากร วิทยาเขต พระราชวังสนามจันทร์ จังหวัดนครปฐม.

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2. นางสาวนงนุช กำลังแพทย์ ตำแหน่งนักวิทยาศาสตร์ ระดับ 6

วุฒิการศึกษา วทบ. (พืชศาสตร์) สถาบันเทคโนโลยีการเกษตรแม่โจ้

วทม. (วิทยาศาสตร์สิ่งแวดล้อม)

ปัจจุบัน นักวิทยาศาสตร์ ระดับ 6 ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยศิลปากร วิทยาเขตพระราชวังสนามจันทร์ จังหวัดนครปฐม.

ผลงานตีพิมพ์

คณิสส์ เสงี่ยมสุนทร, **นงนุช กำลังแพทย์,** เรณู เวชรัชต์พิมล (2548) ความเป็นพิษของสารสะเดาสกัดที่มีผลต่อสารพันธุกรรมในเซลล์เพาะเลี้ยงของหนู (L929) การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 31 (วทท.11) วันที่ 18-20 ตุลาคม 2548 ณ เทคโนธานีมหาวิทยาลัยเทคโนโลยีสุรนารี จ.นครราชสีมา

ชินรัตน์ แจ้งแสงฟ้า, **นงนุช กำลังแพทย์,** เรณู เวชรัชต์พิมล (2548) การเปรียบเทียบวิธีประเมินความเป็นพิษของทองแดงต่อเซลล์เพาะเลี้ยงของหนูเม้าส์. การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 31 (วทท.11) วันที่ 18-20 ตุลาคม 2548 ณ เทคโนธานีมหาวิทยาลัยเทคโนโลยีสุรนารี จ.นครราชสีมา

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Sunlight decreased genotoxicity of azadirachtin on root tip cells of *Allium cepa* and *Eucrosia bicolor*

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ABSTRACT

Utilization of neem plant (*Azadirachta indica* A. Juss) extract for pest control in agriculture has raised concerns over contamination by the residues to the environment. Such residues, particularly azadirachtin (Aza), may cause deleterious effect to non-target organisms. This investigation was conducted to find out if Aza could be inactivated through exposures to sunlight. Activity of Aza was assessed as its ability to cause cytotoxic and genotoxic effects in the forms of nuclei abnormality and chromosome aberration as measured by mitotic index (MI) and mitotic aberration (MA). Varying concentrations of Aza were tested on *Allium cepa* and *Eucrosia bicolor*. It was found that the MI of all root tip meristematic cells of *A. cepa* and *E. bicolor* treated with 0.00005%, 0.00010%, 0.00015%, and 0.00020% (w/v) Aza-containing neem extract for 24 h, were significantly lower than the controls. Complementary to the lower levels of MI, the Aza-treated groups showed higher MA levels in all cases investigated. Furthermore, the decreasing levels of MI and the increasing levels of MA related well with the increasing concentration of Aza. Microscopic examination of root tip meristematic cells revealed that the anomaly found most often were mitotic disturbances and chromosomal bridges. Exposures of 0.00020% (w/v) Aza to sunlight for 3 days and 7 days decreased Aza ability to induce cytotoxicity and genotoxicity, both in terms of MI and MA, to root tip meristematic cells in *A. cepa* and *E. bicolor*. Photodegradation of Aza upon exposure to direct sunlight was confirmed by HPLC. The study implicates that Aza would unlikely cause long term deleterious effects to the environment since it would be inactivated by sunlight.

1. Introduction

Contamination by toxic agents in the environment, which is a consequence of overuses of synthetic pesticides and herbicides, has become a serious problem in many agricultural countries. Replacing those chemicals with nature friendly substances to reduce harm on environments, would presumably be an effective solution to the problem. Biochemical extracts from numerous plant species have been investigated for their efficacies against pests both in the field and on seed stock (Boeke et al., 2004). The extract from different parts of one plant species, the neem plant (*Azadirachta indica* A. Juss.), has become a widely used biopesticide. Neem extract contains more than 100 active chemicals, notably azadirachtin (Aza), salannin and meliantriol, which have been demonstrated to have insecticidal properties against a broad spectrum of insects (Vietmeyer, 1992). The most active secondary metabolite in neem is the triterpenoid Aza, which has a chemical structure similar to ecdysone, an insect steroid hormone involved in metamorphosis (Singh et al., 1993).

Although a neem extract has a potential to be used as an alternative biopesticide, the results of many studies showed that they also caused both positive and negative biological effects in human and non-target animals. For examples, taking unprocessed neem leaves at a given dose could render an anti-diabetic effect, while taking neem at another dose caused severe renal failure (Alam et al., 1990; Kadiri et al., 1999). Sinniah et al. (1982) reported that people who took 12 ml of neem oil for 2 days showed severe toxic symptoms. Neem kernel powder (75 and 100 mg/kg of body weight) fed to sheep showed a good anthelmintic effect against intestinal nematodes. However, sheep were adversely affected when fed with 100 g/cap of leaves (Ali and Salih, 1982; Ahmed et al., 1994). In addition, experiments in murine showed that neem leaves extract induced many deleterious effects such as abnormality of bone marrow cells, altered chromosome structure and chromosome numbers in spermatocytes, failure of chromosome synapsis during metaphase I, deformation of sperm's head, and reduction of sperm numbers (Khan and Awasthy, 2001, 2003). These undesirable effects have

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raised concerns whether the use of neem-derived products as pesticides is safe. Residues left behind after the application of neem extract to crops may cause harms to people as well as to other animals in the ecosystem.

To evaluate the cytotoxicity and genotoxicity of known and unknown toxic agents, more than 200 short-term assays have been developed (Grant, 1994; Soliman, 2001; Akinboro and Bakare, 2007). Among those assays, mitotic cell division inhibition and chromosome aberration induction have been widely used as indicators of cytotoxicity and genotoxicity. Consistent with many reports, *Allium cepa* assay, which is established by the international program on chemical safety and the World Health Organization (WHO), is one of the most effective and sensitive methods for mutagent testing (Rank and Nielsen, 1994). Thailand is a country which has been actively promoting the use of neem extract as pest control. Together with that campaign there has been an environmental concern over whether the use of Thai commercial neem extract at the manufacturer's recommended dose of 0.00015–0.00025% (w/v) is toxic to plants and animals. Farmers are also interested in knowing what factors would affect the toxicity of Aza, and how long would toxicity of Aza last in the normal circumstances of use. It was found that the persistence of active metabolites in neem extract depend on many factors such as temperature during storage or application, acidic or basic condition of solution and neem extract formulation (Yakkundi et al., 1995; Stark, 1996; Kongkathip and Sombutsiri, 1996). Additionally, Javed et al. (2007) reported that neem extract could persist in soil up to 4 months. It, therefore, became an objective of this study to assess genotoxicity of Aza after exposing the chemical to sunlight for certain periods of time. Apart from using *A. cepa* assay as a standard plant for testing cytotoxicity and genotoxicity we also aimed at exploring the use of *Eucrosia bicolor* as an alternative testing plant species.

2. Materials and methods

2.1. Testing solutions

Varying concentration of Aza testing solutions, namely, 0.00005%, 0.00010%, 0.00015%, and 0.00020% (w/v) Aza were prepared from commercial 0.1% (w/v) Aza-containing neem extract stock solution. Actual concentration of Aza in the 0.00005%, 0.00010%, 0.00015%, and 0.00020% (w/v) Aza testing solutions were estimated by HPLC and found to contained Aza at the levels of 8.30, 16.61, 24.9 and 33.21 mg/L, respectively. Controls were set up similarly except that distilled water was used instead of Aza.

The study on the effect of sunlight on the activity of Aza was carried out by first exposing 0.00020% (w/v) Aza in flat-shaped bottles to direct sunlight for 3 and 7 days before the solutions were used for the study. The sunlight-unexposed 0.00020% (w/v) Aza solutions were the solutions kept in dark before being used as controls (0 day sunlight exposed).

Table 1
Phase index (‰PI) and Mitotic index (‰MI) of root tip cells treated with 0.00000%, 0.00005%, 0.00010%, 0.00015% and 0.00020% (w/v) Aza-containing neem extract for 24 h.

Plant species	Neem extract conc. (%)	Phase index (‰ PI)					Mitotic index (‰MI)
		PI-I	PI-P	PI-M	PI-A	PI-T	
A. cepa	0.00000	907 ± 2.5^{a}	46 ± 4.2^{c}	15 ± 1.8^{b}	9 ± 1.1^{b}	23 ± 2.3^{c}	92.6 ± 2.5^{c}
	0.00005	954 ± 8.2^{c}	25 ± 2.9^{ab}	6 ± 2.2^{a}	5 ± 1.4^{ab}	10 ± 3.3^{ab}	45.8 ± 8.2^{ab}
	0.00010	973 ± 13.2^{c}	14 ± 5.7^{a}	5 ± 2.7^{a}	2 ± 1.9^{a}	5 ± 2.9^{a}	26.8 ± 13.2^{a}
	0.00015	924 ± 20.0^{ab}	38 ± 12.5^{a}	13 ± 3.9^{ab}	7 ± 1.9^{ab}	18 ± 6.3^{bc}	76.2 ± 20.0^{ab}
	0.00020	951 ± 2.9^{bc}	23 ± 1.8^{a}	8 ± 1.6^{ab}	5 ± 0.9^{ab}	13 ± 1.2^{abc}	49.2 ± 2.9^{ab}
E. bicolor	0.00000	930 ± 3.3^{a}	24 ± 2.4^{b}	22 ± 0.5^{d}	13 ± 1.2^{b}	11 ± 0.6^{c}	69.8 ± 3.3^{b}
	0.00005	952 ± 4.9^{b}	15 ± 3.3^{a}	14 ± 1.9^{bc}	8 ± 1.2^{a}	11 ± 1.0^{bc}	47.2 ± 4.9^{a}
	0.00010	954 ± 2.9^{b}	16 ± 2.1^{a}	15 ± 0.8^{c}	9 ± 1.8^{a}	6 ± 0.9^{a}	45.8 ± 2.9^{a}
	0.00015	962 ± 4.7^{b}	14 ± 2.6^{a}	11 ± 1.4^{ab}	$6. \pm 1.2^{a}$	6 ± 1.0^{a}	37.4 ± 4.7^{a}
	0.00020	963 ± 0.4^{b}	12 ± 1.8^{a}	9 ± 1.2^{a}	7 ± 1.1^{a}	8 ± 0.8^{ab}	36.2 ± 0.4^{a}

PI = phase index of interphase (-I), prophase (-P), metaphase (-M), anaphase (-A) and telophase (-T).Values were expressed as ‰PI or ‰MI ± standard deviation.

[a,b,c] Values for each parameter in the same row, in each plant species, followed by the same letter are not significant different ($P < 0.05$.).

Table 2
Chromosome aberrations and Mitotic aberration index (‰MA) of root tip cells treated with 0.00000%, 0.00005%, 0.00010%, 0.00015% and 0.00020% (w/v) Aza-containing neem extract for 24 h.

Plant species	Neem extract conc. (%)	Chromosome aberration (‰)						Mitotic aberration index (‰MA)
		Bridge	Disturb	Laggard	Fragment	MN	Bi	
A. cepa	0.00000	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0^{a}
	0.00005	0.6 ± 0.4	0.2 ± 0.2^{a}	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.4	0.0 ± 0.0	1.4 ± 0.7^{a}
	0.00010	0.0 ± 0.0	0.6 ± 0.6^{a}	0.0 ± 0.0	0.2 ± 0.2	0.2 ± 0.2	0.0 ± 0.0	1.0 ± 0.4^{a}
	0.00015	0.8 ± 0.6	1.2 ± 0.6^{ab}	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	0.2 ± 0.2	2.8 ± 1.0^{ab}
	0.00020	0.6 ± 0.4	2.8 ± 1.0^{b}	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.6	0.2 ± 0.2	4.4 ± 1.7^{b}
E. bicolor	0.00000	0.0 ± 0.0	0.0 ± 0.0^{a}	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.2 ± 0.2	0.4 ± 0.2^{a}
	0.00005	0.0 ± 0.0	2.8 ± 0.9^{b}	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.6 ± 0.4	3.6 ± 0.9^{b}
	0.00010	0.0 ± 0.0	3.6 ± 0.4^{b}	0.0 ± 0.0	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	4.2 ± 0.6^{b}
	0.00015	0.0 ± 0.0	2.8 ± 0.4^{b}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.58	4.0 ± 0.5^{b}
	0.00020	0.0 ± 0.0	3.0 ± 0.3^{b}	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	3.4 ± 0.5^{b}

MN = Micronucleated cells, Bi = Binucleated cells Values were expressed as ‰ (chromosome aberration) or ‰MA ± standard deviation.

[a,b,c] Values for each parameter in the same row, in each plant species, followed by the same letter are not significant different ($P < 0.05$).

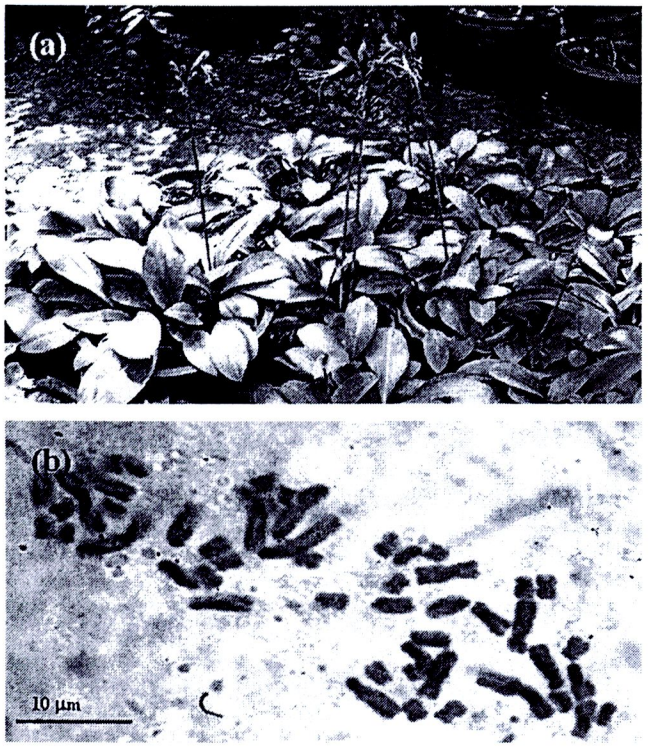

Fig. 1. *Eucrosia bicolor* showing some plants with flowers (a), and Metaphase chromosome plate from root tip cell with $2n=50$ (b).

2.2. Preparation of plants for cytotoxicity and genotoxicity assays

Two plant species, *A. cepa* and *E. bicolor*, were used for cytotoxicity and genotoxicity testing. Healthy bulbs were prepared for use by first removing outer scales and existing roots. The bulbs were then placed in small jars having their basal ends dipped in distilled water and kept at room temperature. When the newly emerged roots were 1–2 cm in length, the plants were treated for 24 h with testing solutions (5 bulbs/concentrations). Recovery of all treated plants was done by placing plants with root tips submerged in distilled water for 24 h before root tips were removed for cytogenetic study.

2.3. Cytogenetic study

The cytotoxicity and genotoxicity of Aza on cell division and chromosome abnormality were assessed based on mitotic index (MI) and mitotic aberration (MA), respectively. After a recovery in distilled water for 24 h, treated root tips were fixed in Carnoy I solution (3:1 ethanol/glacial acetic acid; v/v) and stored overnight in a refrigerator (4 °C). Root tip cells were prepared for examination under a light microscope by first pre-treating in 1 M HCl at 60 °C for 5 min. Acid pretreated root tips were then washed with distilled water and stained with acetoorcein or acetocarmine for 5 min before squashing. In all cases, 5000 cells (1000 cells/slide) from 5 bulbs were examined under a light microscope at 400 times magnification. Mitotic index (MI), phase index (PI), and mitotic aberration (MA) were calculated as the proportion of dividing cells, cells in each division phase, and abnormal cells per 1000 examined cells, respectively (Grant, 1982; Fiskesjo, 1985, 1997; Soliman, 2001; Hala et al., 2007).

2.4. High performance liquid chromatography of Aza-containing neem extract exposed to sunlight.

The contents of Aza in the 0.00020% (w/v) Aza solutions prepared from commercial 0.1% (w/v) Aza-containing neem extract exposed to sunlight for 0 day, 3 days, and 7 days were estimated by a high performance liquid chromatography (HPLC)-based method. Azadirachtin standard was obtained from Sigma-Aldrich

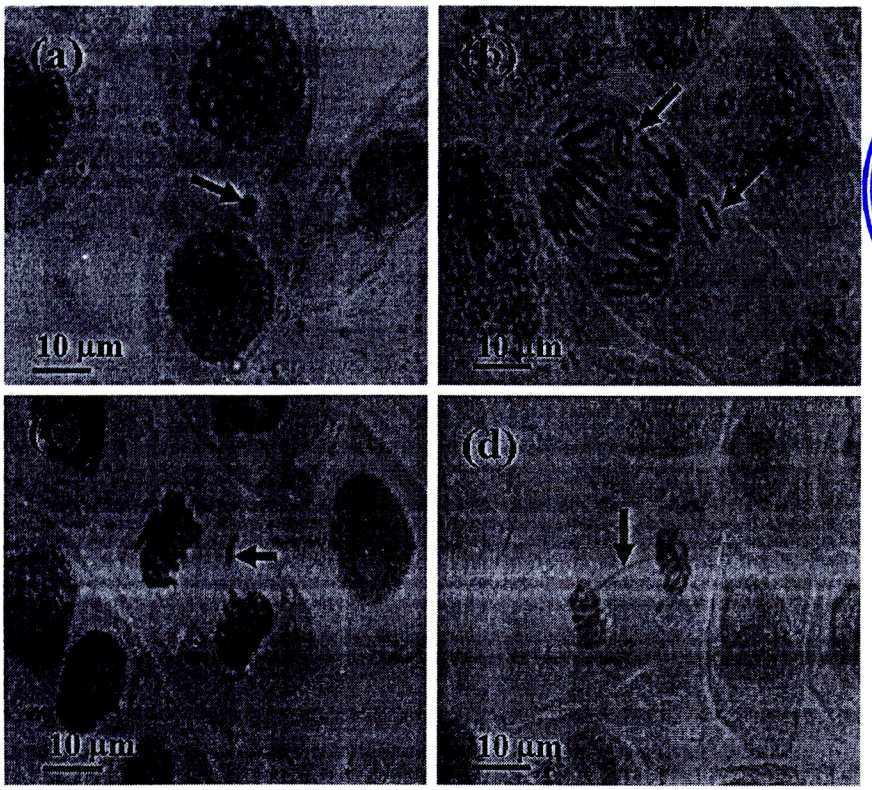

Fig. 2. Abnormality of interphase nucleus and mitotic cells in treated root tips from *Allium cepa*. Occurences of abnormalities were observed (arrowheads) in the case of micronucleus (a), disturbed anaphase (b), laggard chromosome (c), and telophase bridge (d).

(USA). The analysis was conducted using a Pursuit C18 column (5 μm, 250 × 4.6 mm^2 I.D.) fitted to the Agilent 1100 Series HPLC System equipped with 1100 Binary Pump, and the 1100 Diode Array. The samples (20 μl) were injected into the HPLC using an autoinjector. The mobile phase used was acetonitrile–water (40:60; v/v), which was run at a flowrate of 1 ml min^{-1} while the UV signals were recorded at 210 nm (Kaushik, 2002). The standard Aza eluted at a retention time of 10.5 min. The chromatograms and data were acquired and processed with the HP Chemstation Data System (Scientific Equipment Center, Kasetsart University, Thailand).

2.5. Statistical analysis

The data were analyzed using ANOVA and means compared with Duncan's multiple range test (DMRT) under a 95% confidence interval (α 0.05).

3. Results

3.1. Effects of azadirachtin on MI and MA of root tip cells

Aza clearly inhibited root tip cell division as evident in the lowering of MI in all treated groups of *A. cepa* and *E. bicolor*. The inhibitory effects of Aza were dose-dependent in the case of *E. bicolor* but fluctuated among doses in the case of *A. cepa* (Table 1). In all cases, decreasing levels of MI in treated groups were accompanied by higher levels of phase index (PI) of interphase cells (PI-I) but lower levels of mitotic phase-index, which comprised the phase index of prophase (PI-P), metaphase (PI-M), anaphase (PI-A) and telophase (PI-T). Root tip cells of *A. cepa* and *E. bicolor* exhibited similar responses to Aza regarding MI and PI (Table 1).

Aza at all concentrations significantly increased MA values of root tip cells in *E. bicolor* ($p < 0.05$). Even though treatment with Aza at all concentrations resulted in increases of MA in root tip cells of *A. cepa* the effects were statistically significant only at 0.00015% and 0.00020% of Aza (Table 2). It is also evident that *E. bicolor* is likely a good candidate for an alternative testing plant species. The plant has an interesting feature in that the chromosomes are large (Fig. 1) thus facilitates chromosome visualization as well as the screening for chromosome abnormalities. Microscopic examination of root tip cells revealed six major types of nucleus abnormality and chromosome aberration (Table 2). These were micronucleus, metaphase or anaphase disturb, laggard, bridge, fragmented and binucleated cells (Fig. 2). The most frequent type of aberration found was metaphase or anaphase disturb in both *A. cepa* and *E. bicolor*.

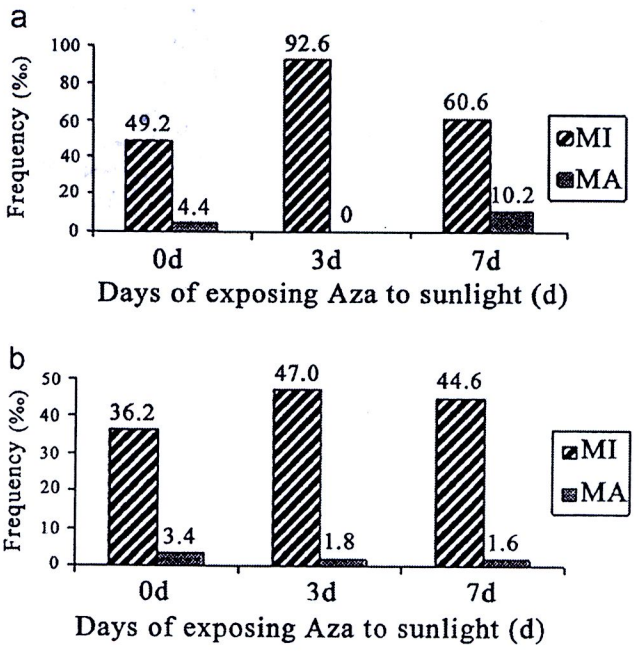

Fig. 3. Cytotoxity and Genotoxicity of sunlight-exposed 0.00020% (w/v) Aza on root tip cells of *Allium cepa* (a) and *Eucrosia bicolor* (b) measeured as mitotic index (‰MI) and mitotic aberration index (‰MA).

3.2. Effects of exposing azadirachtin to direct sunlight

Exposing 0.00020% (w/v) Aza to direct sunlight for either 3 days or 7 days resulted in marked reductions in cytotoxicity and genotoxicity in terms of the effects on cell division and chromosome abnormality. The MI values of treated groups of *A. cepa* and *E. bicolor* increased substantially comparing to the sunlight-unexposed group shown as day 0 (Fig. 3.). Supporting the increases in mitotic index, the MA values of the two plant species showed similar lowering trends. This is particularly true in the case of *E. bicolor* where the MA decreased from 3.4 in 0 day group to 1.8 then to 1.6 in 3 day and 7 day groups, respectively (Fig. 3.). In the case of *A. cepa* the MA decreased from 4.4 in day 0 group to no MA at all in 3 day group. However, the 7 day group showed substantial increases in MA frequency.

The reduction of cytotoxicity and genotoxicity of Aza exposed to sunlight was confirmed by HPLC analysis. The relative percentage reductions of Aza based on the Aza content of the control (0 day sunlight exposed 0.00020% (w/v) Aza solution), were 17.89% and 51.27% for the 3 day, and 7 day sunlight exposed solutions, respectively (Fig. 4).

4. Discussion

This present investigation was conducted to find out if Aza could be inactivated through exposures to sunlight. Cytotoxicity and genotoxicity of Aza was assessed using *A. cepa* assay where another parallel test using *E. bicolor* was employed to confirm the results. The decreases in MI in all Aza concentration groups of *A. cepa* and *E. bicolor* are in good agreement with the work done on *A. cepa* by Soliman (2001). It was shown by Soliman (2001) that the extract from neem leaves, kernels and seed coats inhibited mitotic cell division in *A. cepa* root tip cells exposed to neem extract for 24 h. In our study the decreases of MI seen in all Aza-treated groups were always accompanied by higher levels of PI-I and lower level of mitotic phase-index. This finding implied that the reduction of MI might be a result of inhibition of the cell cycle, thus slowing down the progression through mitosis. More cells were arrested at the interphase stage which consequently led to the reduction in rates of cell division. Considering the three sub-phases of interphase, there are a number of reports showing that only synthetic phase (S) or gap2 phase (G2) which was frequently disturbed by genotoxic substances (Van't Hoff, 1968; Webster and Davidson, 1969; Salehzadeh et al., 2003). According to Macleod (1969) and Brunori (1971), disturbance of interphase stage at S or G2 could occur through sub-phases inhibition or increasing of phase duration. It was suggested that the mechanism of Aza-mediated interphase cell arrest could be the result of up or down-regulation by specific proteins which control the transition of cell cycles (Kumar et al., 2006).

Disturbed or non-congression metaphase and anaphase, the most frequent type of aberrations occurred in both *A. cepa* and *E. bicolor*, could be caused by destruction of the spindle apparatus. This phenomenon usually results in unequal distribution of chromosome complements (Rosenkranz and Klopman, 1995a,1995b). Salehzadeh et al. (2003) and Wang et al. (2006)

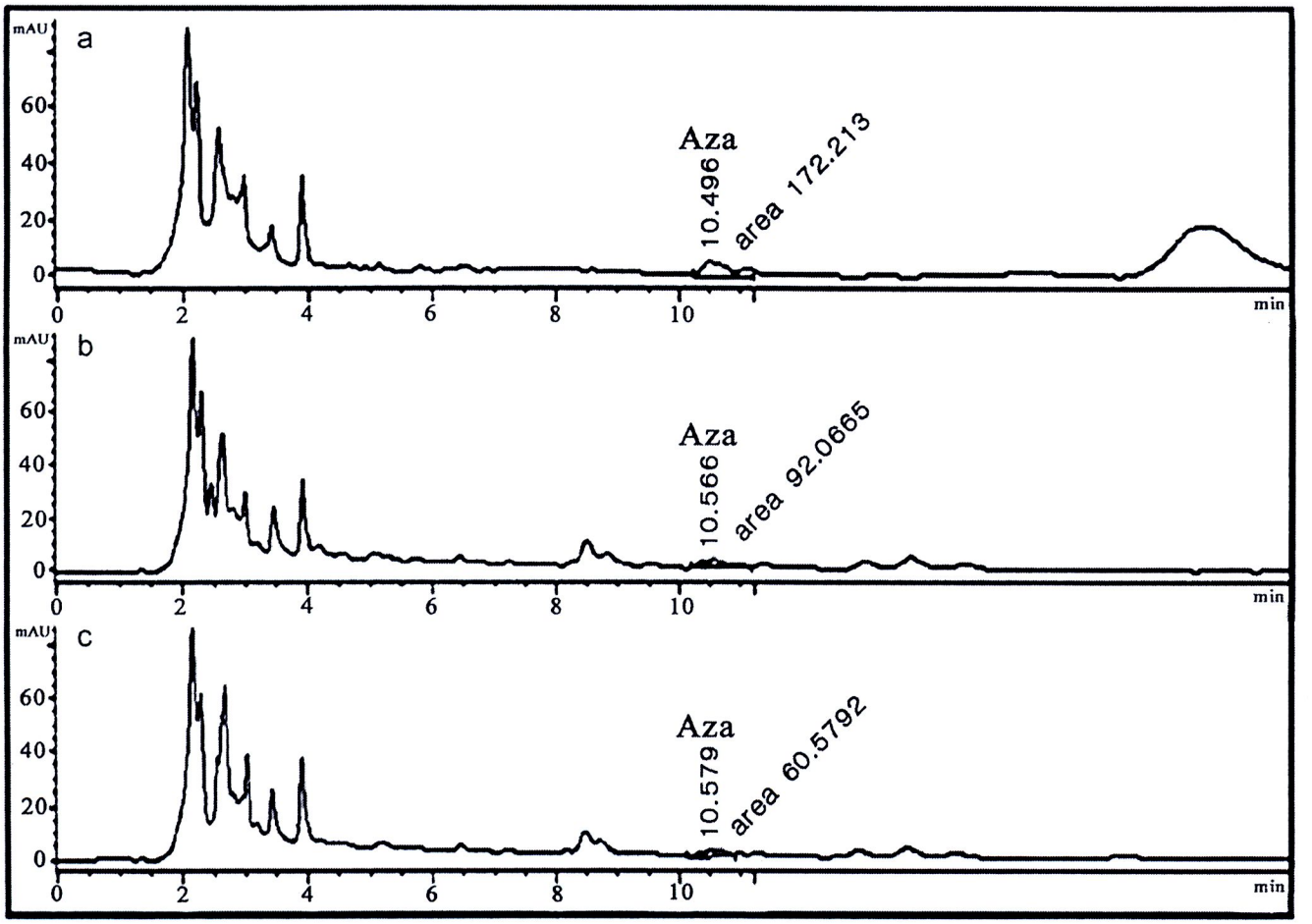

Fig. 4. Chromatograms of 0.00020% (w/v) Aza solution after direct exposed to sunlight for 0 day (a), 3 days (b) and 7 days (c) obtained by using HPLC.

reported that Aza has a chemical characteristic similar to colchicine, an anti-mitotic metabolite affecting synthesis and depolymerization of spindle fibers. It was then suggested that Aza could possibly inhibit mitotic spindle formation, or could induce deformation of the spindle apparatus. A consequence would be an unequal distribution of chromosomes, thus the occurrence of mitotic disturbance found in this research. However, the prominent abnormality types showed a trend relating to the concentration of Aza. This view was supported by the results of Soliman (2001) who showed, for example, that the bridge was the most common abnormality observed when treating root tip cells with varying concentrations of Aza prepared from suspension stock (100 g/L). Moreover, genetic background of each plant would also play a key role in responding to Aza thus give rises to mitotic and chromosome abnormality.

The increases in MI which accompanied by the decreases of MA after Aza was exposed to direct sunlight indicated that Aza could be degraded by a common factor normally presented in the field, the sunlight. Our bioassay results indicating reductions on Aza-causing genotoxicity agree well with the HPLC investigation showing decreasing Aza levels after exposing to sunlight. Our finding was in line with the report by Caboni et al. (2006) who showed that Aza was very sensitive to sunlight, degrading rapidly with half-life ranging from 5.5 to 11.3 h. Exposure of Aza spread on thin film to direct UV light has been reported to reduce the half-life of the compound to 48 min (Dureja and Johnson, 2000). The susceptibility of Aza in neem seed to photodegradation after storing the seeds in daylight condition was also reported by Yakkundi et al. (1995). These investigators found that Aza content was reduced to about 55% of the original level. Another factor, types of solvents used in Aza formulation, was shown to decrease half-life of Aza (Stark, 1996). Additionally, the storage of neem crude extracts at room temperature instead of keeping in a refrigerator could accelerate the rate of Aza decomposition (Kongkathip and Sombutsiri, 1996). It can, therefore, be inferred that degradation of Aza after an exposure to direct sunlight could be caused by the common factors associated with the sunlight, namely, the high temperature and the UV radiation. The results presented here indicated that commercial 0.1% (w/v) Aza-containing neem extract in water, at the levels commonly used for controlling pests, could be rapidly degraded by sunlight thus reducing the effect of Aza to a non-toxic level within a few days.

5. Conclusions

Aza-containing neem extract decreased mitotic index in root tip cells of *A. cepa* and *E. bicolor*. Cytotoxicity and genotoxicity caused by Aza was evident in the forms of nucleus abnormality and chromosome aberration which led to higher mitotic aberration in the plants root tip cells. Exposure of Aza to direct sunlight for 3 days and 7 days practically deactivated Aza in terms of its cytotoxicity and genotoxicity. The findings support

the results from HPLC analysis showing that the amount of Aza decreased after exposed to sunlight under the same conditions.

Acknowledgments

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Screening for Plants Sensitive to Heavy Metals Using Cytotoxic and Genotoxic Biomarkers

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ABSTRACT

Cytotoxic and genotoxic sensitivity to heavy metals were investigated in five plant species using a mitotic index (MI) and mitotic aberration (MA) as biomarkers. The results showed that *Eucrosia bicolor* and *Allium cepa* was more sensitive to cadmium toxicity at concentrations ranging from 0.02 to 0.09 mg.L^{-1} of $CdCl_2$ than *A. cepa* var. ascolonicum, *Zephyranthes rosea* and *Wedelia triloba*. A decrease in the ‰MI and an increase in the ‰MA were found in *E. bicolor* as early as 24 hr after Cd treatment. Testing with lead (Pb), copper (Cu) and arsenic (As) showed that *E. bicolor* and *A. cepa*, to be sensitive to toxicity by Pb at the lowest dose of 0.2 mg.L^{-1} of $Pb(NO_3)_2$. Cyto-genotoxicity from Pb was also dose dependent. *E. bicolor* showed greater sensitivity than *A. cepa* in testing for Cu sensitivity. The lowest dose (1.00 mg.L^{-1}) of $CuCl_2$ caused a significant decrease in the ‰MI only in *E. bicolor*. The highest frequency of chromosome aberration type induced by Cu was different from that of Pb. *E. bicolor* was less sensitive to As cyto-genotoxicity compared with *A. cepa*. Only at 10.00 mg.L^{-1} of $NaAsO_2$ could a significant decline in the ‰MI and an increase in the ‰MA be observed in *E. bicolor*, whereas the lowest dose which affected the ‰MI and the ‰MA of *A. cepa* was 0.25 mg.L^{-1}. This is the first report which has studied and explored the potential of *E. bicolor* for use as a heavy metal cytotoxic and genotoxic bioindicator.

Keywords: *Allium cepa* test, *Eucrosia bicolor,* genotoxicity, heavy metals, mitotic index

INTRODUCTION

The widespread contamination of heavy metals in the environment has become a serious problem in many countries. Pollution from toxic metals has progressively affected living organisms in ecosystem including the top consumers, human beings. Bioaccumulation at higher doses of heavy metals, such as cadmium (Cd), lead (Pb), copper (Cu) and arsenic (As), through the food chain carries risks in many forms to humans including neurotoxicity, hepatotoxicity, nephrotoxicity, mutagenicity and carcinogenicity (Filipic and Hei, 2004; Graham-Evans *et al.*, 2004; Benavides *et al.*, 2005; Depault *et al.*, 2006; Islam *et al.*, 2007). Emphasis on heavy metals toxicity has been directed to their abilities to induce free radical formation and biomethylation as well as altering gene regulation (Durham and Snow, 2006). High Cd toxicity at low concentrations was a result of its ability to dissolve easily in water (An, 2004). Toxicity of Cd to animals and other organisms has been shown by a number of investigations and it has been shown to reduce plant growth,

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photosynthesis and chlorophyll content as well as to induce oxidative stress (Degraeve, 1981; Li and Xiong, 2004; Wang and Zhou, 2005; Mishra *et al.*, 2007). In addition to the phyto-toxic effects, Cd was also shown to inhibit plant cell proliferation, to alter RNA synthesis and to induce chromosomal aberrations similar to those observed in animal cells (Rosas *et al.*, 1984; Toppi and Gabbrielli, 1999; Unyayar *et al.*, 2006). Lead is known to be toxic to various systems, for instance, the reproductive system, the liver and kidney system and also the immune system (Silbergeld *et al.*, 2000). Pb toxicity stems from its ability to bind strongly to different types of biomolecules such as amino acids, DNA and RNA, and several enzymes, thus interfering with many metabolic pathways. Arsenic shows the same trend as lead. It is an effective mitotic poison due to its ability to induce spindle disturbances (Patra *et al.*, 2004). Exposure of human leukemia cells to As led to an increase in the number of DNA single-strand breaks in a dose-dependent manner (Yedjou and Tchounwou, 2007). Cytotoxicity and genotoxicity of copper in human cells have been reported as the result of reactive oxygen species generation, oxidative DNA base modifications and DNA strand breaks (Schwerdtle *et al.*, 2007; Kashanian *et al.*, 2011).

Plant bioassays have been recommended by the United Nations Environment Program (UNEP), the World Health Organization (WHO) and the US Environment Protection Agency (US-EPA), as a highly efficient system for screening of cytotoxic and genotoxic chemicals (Grant, 1999; Ma, 1999). The exploitation of plants as cyto-genotoxic bioindicators has been well recognized, such as in the *Allium* genotoxicity test or the *Allium* test, due to the use of *Allium cepa* (Levan, 1938; Kristen, 1997). It is also recognized that plants species which are easy to culture and handle, have large chromosomes and are sensitive to a wide range of toxicants, are reliable plants for using as cytotoxic and genotoxic bioindicators. This is in line with Grant (1982), who reported that *Allium cepa* (2n=16) gave a positive response to some 76% of the chemicals that were tested. Assays with *Lycopersicum esculentum* (2n=24) showed positive responses to 19 out of 21 tested chemicals (Grant and Owens, 2002). Chromosome aberrations in *Zea mays* (2n=20) were induced by at least 78 tested chemicals (Grant and Owens, 2006). In addition to the sensitivity, simplicity and efficiency in using higher plants for bioassays, there was also an excellent correlation between the plant testing system and other test systems, including the mammalian cell system (Grant, 1978; Fiskesjo, 1985; Kristen, 1997; Saxena *et al.*, 2005; Dimitrov *et al.*, 2006). Many reports showed that toxicity from heavy metals, air pollutants, many chemical compounds and physical and chemical mutagens can be assessed with micronucleus assay, chromosome aberration assay and gene mutation assay (Kristen, 1997; Kim *et al.*, 2003; Yi and Meng, 2003; Grant and Owen, 2006). In addition, different forms of anomalies can occur in plants, such as mitotic abnormality, chromosome aberration and the appearance of micronuclei, which can all be used as cytotoxic and genotoxic biomarkers. (Cotelle *et al.*, 1999; Grant and Owen, 2001; Uhl *et al.*, 2003; Fusconi *et al.*, 2006; Hala *et al.*, 2007; Yildiz *et al.*, 2009).

The current study was conducted to screen for plants sensitive to heavy metals using cytotoxic and genotoxic biomarkers. In addition to the common onion (*Alllium cepa*) and shallot (*Allium cepa* var. ascolonicum), screening used three other plant species—namely, Peruvian lily (*Eucrosia bicolor*), rain flower (*Zephyranthes rosea*), and climbing wedelia (*Wedelia triloba*)—by treating the plants with various types of heavy metals. The frequency of mitotic cell division and chromosome aberration were subsequently examined. The sensitive plants selected from this experiment were explored for their potential as bioindicators for assessing heavy metals contamination in an ecosystem.

MATERIALS AND METHODS

Plant materials

Heavy metal sensitivity was investigated in five plant species—namely, common onion (*Allium cepa*) which has been well-accepted as a common testing plant for genotoxic assay, shallot (*Allium cepa* var. ascolonicum), Peruvian lily (*Eucrosia bicolor*), rain flower (*Zephyranthes rosea*), and shoots of climbing wedelia (*Wedelia triloba*). All plants were obtained from the Silpakorn University greenhouse.

Testing chemicals

Four types of heavy metals in the form of solutions of cadmium chloride ($CdCl_2$. $2.5H_2O$, Sigma Aldrich Chemical Co.) at 0.00, 0.02, 0.03, 0.06 and 0.09 $mg.L^{-1}$, sodium arsenite ($NaAsO_2$, Fluka Chemical) at 0.00, 0.25, 1.00 and 10.00 $mg.L^{-1}$, lead nitrate ($Pb(NO_3)_2$, Sigma Aldrich Chemical Co.) at 0.00, 0.20, 25.00 and 200.00 $mg.L^{-1}$, and copper chloride ($CuCl_2$, Univar USA Inc.) at 0.00, 1.00, 2.00 and 3.00 $mg.L^{-1}$, were prepared in distilled water. The doses of the testing chemicals were designed to cover the range from low permissible concentrations to high nonpermissible concentrations. These high nonpermissible concentrations were based on the industrial standards for effluent (Thai Ministry of Science, Technology and Environment, 1996).

Screening for heavy-metal-sensitive plants using mitotic and chromosome aberration assay

The heavy-metal-sensitive plant species were initially screened using a strong heavy metal mutagen, Cd (Gomes *et al.*, 2008). Decreases in mitotic cell division and increases in chromosome aberration were examined and used as cytotoxic and genotoxic biomarkers. At least five healthy bulbs, without older outer scales and existing roots, of *A. cepa*, *A. cepa* var. ascolonicum, *E. bicolor*, *Z. rosea* , and five shoots of *W. triloba* were placed in small bottles with their basal ends dipped in distilled water and kept at room temperature. When the newly emerged roots were 1-2 cm in length, they were treated with different concentrations of cadmium (0, 0.02, 0.03, 0.06 and 0.09 $mg.L^{-1}$) for 24 hr, followed by leaving in water for 48 hr to recover.

After recovery, the treated roots were fixed in Carnoy I (3:1 ethanol/glacial acetic acid; v/v) and stored overnight in a refrigerator (4 °C). A squash technique was used in the preparation of specimens for microscopic examination. Initially, fixed root tips were hydrolyzed in 1 N HCl at 60 °C for 5 min, washed with distilled water and stained with aceto-orcein or aceto-carmine for 5 min. For each treatment, 5,000 cells (1,000 cells/slide) from five bulbs were examined under a light microscope at 600× magnification. The mitotic index (MI) and mitotic aberration (MA) were calculated from the proportion of dividing cells and abnormal cell per 1,000 examined cells, respectively. (Grant, 1982; Fiskesjo, 1985; Fiskesjo, 1997; Soliman, 2001; Hala *et al.*, 2007).

The effects of different exposure durations

Cadmium-sensitive plants screened by the first experiment were subsequently used to evaluate the effects of different exposure durations to heavy metals. Healthy bulbs with root tips were treated with 0.06 $mg.L^{-1}$ $CdCl_2$ for 24 hr and 48 hr. The frequencies of MI (‰MI) and MA (‰MA) were subsequently determined.

Sensitivities of selected-plants to Pb, Cu and As

Heavy-metal-sensitive plant species selected from the screening test were used as cyto-genotoxic bioindicators for assessing other heavy metal contamination. Healthy root tips of selected plants were exposed to 0.00, 0.20, 25.00 and 200.00 $mg.L^{-1}$ of $Pb(NO_3)_2$, 0.00, 1.00, 2.00 and 3.00 $mg.L^{-1}$ of $CuCl_2$ and 0.00, 0.25, 1.00 and 10.00 $mg.L^{-1}$ of $NaAsO_2$, for 24 hr. Susceptibility to the testing chemicals was evaluated based on the

frequency of mitotic inhibition and chromosome aberration induction, using the same microscopic method as in the screening experiment.

Statistical analysis

The data from the experiments with each tested plant species were statistically analyzed using one-way ANOVA and Duncan's multiple range test to compare differences among treatments. The significant difference was set at the level of $P < 0.05$.

RESULTS

Screening for heavy-metal-sensitive plants using mitotic and chromosome aberration assay

According to the cytotoxic-indicative parameter (‰MI), mitotic depression was observed in all Cd-treated groups. Cadmium clearly inhibited mitotic cell division in the root tip cells of three plant species—namely, *E. bicolor*, *Z. rosea* and *A. cepa*. Responses to Cd toxicity at concentrations ranging between 0.02 and 0.09 mg.L^{-1} $CdCl_2$ occurred in a dose dependent manner as depicted in Figure 1. Susceptibility to Cd toxicity as examined by cyto-genotoxic biomarkers showed significant differences only with *E. bicolor* and *A. cepa* (Figure 1). The higher Cd concentrations promoted greater ‰MA values. On the other hand, the frequency of aberration induced by Cd in *Z. rosea* was not different from the control. Fluctuations in the response to Cd, as well as low sensitivity were found in two plant species—*A. cepa* var. ascolonicum and *W. triloba*.

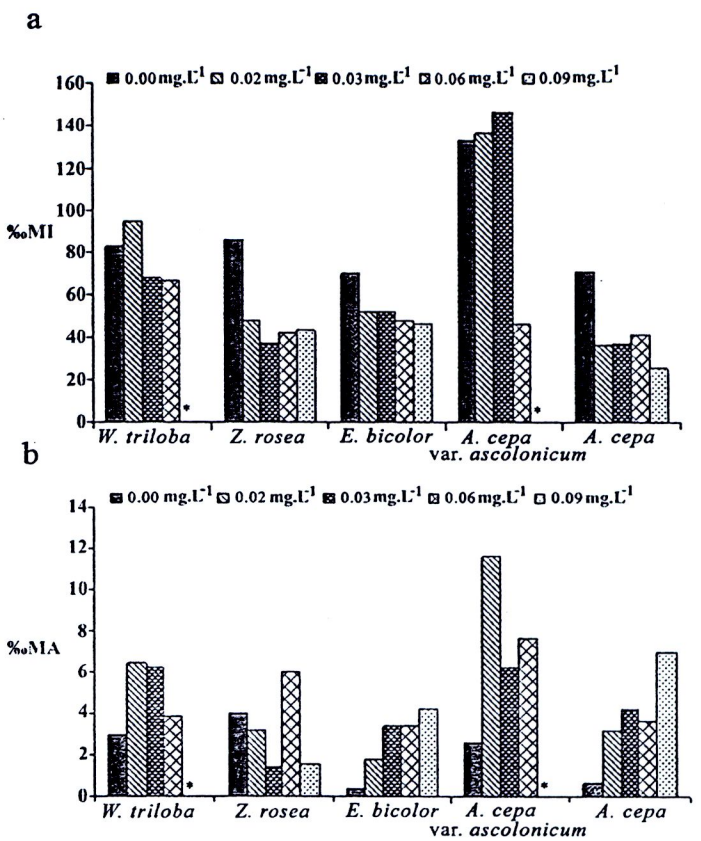

Figure 1 (a) Mitotic index (‰ MI); and (b) Mitotic aberration index (‰ MA) of root tip cells from five plants species treated with 0.00, 0.02, 0.03, 0.06 and 0.09 mg.L^{-1} $CdCl_2$ for 24 hr. The asterisk (*) represents cases of no data due to the death and collapse of root tip cells.

Therefore, only two tested plants (*E. bicolor* and *A. cepa*) were selected based on the sensitivity to Cd as illustrated by the lower ‰MI values and the higher ‰MA values.

Effects of different exposure durations

A longer exposure time (48 hr) to Cd resulted in higher cyto-genotoxicity than the 24 hr treatment, as judged from the decrease in the ‰MI and the increase in the ‰MA (Table 1). Relative mitotic inhibitions were 30% and 41.9% in the root tip cells of *E. bicolor* treated with 0.06 mg.L^{-1} of $CdCl_2$ for 24hr and 48 hr, respectively. Mitotic suppression was clearly seen in *A. cepa* in which cell division was inhibited by up to 65% in the 48 hr treated group.

Sensitivities of *E. bicolor* and *A. cepa* to Pb, Cu and As

Two cadmium-sensitive tested plants, *E. bicolor* and *A. cepa*, were used to assess cytotoxic and genotoxic sensitivity to Pb, Cu and As. It was found that *E. bicolor* was susceptible to toxicity from Pb, like in *A. cepa* (Table 2), but was more sensitive to Cu cyto-genotoxicity than the onion (Table 3). However, *E. bicolor* was slightly less sensitive to the As treatments (Table 4).

Dose-dependent cytotoxicity and genotoxicity of Pb was observed in both plant species, as expressed by the decrease in the ‰MI and the increase in the ‰MA when the $Pb(NO_3)_2$ concentration increased (Table 2). A significant decrease in the ‰MI was found, even in the treated groups exposed to the lowest dose of $Pb(NO_3)_2$. The ‰MI declined from 110.00 to 66.00 and from 103.80 to 94.20 in the case of *A. cepa* and *E. bicolor*, respectively. Six types of aberration were induced by Pb, the highest frequency of aberration was for micronucleus (MN) in both *A. cepa* and *E. bicolor*.

E. bicolor was more sensitive to Cu than *A. cepa*, with a significant decrease in the ‰MI from 114.80 to 104.80, even though it was

Table 1 Mitotic index and mitotic aberration index of *Allium cepa* and *Eucrosia bicolor* root tip cells treated with 0.0 mg.L^{-1} and 0.06 mg.L^{-1} of $CdCl_2$ for 24 hr and 48 hr.

Plant species	Test concentration (mg.L^{-1})	Mitotic index[a]		Mitotic aberration index[a]	
		24 hr	48 hr	24 hr	48 hr
A. cepa	0.0	71.0± 3.09	104.4±3.09	0.6±0.40	2.8±0.66
	0.06	41.4± 4.41	36.2±2.03	3.6±0.87	4.4±1.20
	Relative mitotic inhibition (%)	41.69	65.33		
E. bicolor	0.0	69.0±3.33	79.2±3.92	0.4 ± 0.24	0.0±0.00
	0.06	48.2±3.12	35.0±2.58	3.4 ± 0.92	4.4±0.87
	Relative mitotic inhibition (%)	30.0	41.9		

[a] =Values expressed as mean ± SD.

Table 2 Mitotic index, chromosome aberrations and mitotic aberration index of *Allium cepa* and *Eucrosia bicolor* root tip cells treated with various concentrations of $Pb(NO_3)_2$ for 24 hr.

Plant species	Test concentration ($mg.L^{-1}$)	Mitotic index	Chromosome aberration (‰)						Mitotic aberration index
			Bridge	Disturbed	MN	Laggard	Fragment	Sticky	
A. cepa	0.0	110.0±3.52^{c}	0.4±0.25^{a}	0.0±0.00^{a}	0.6±0.40^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.2±0.58^{a}
	0.2	66.0±5.26^{b}	0.0±0.00^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.4±0.25^{a}
	25.0	58.0±4.47^{b}	0.2±0.20^{a}	0.6±0.40^{a}	6.4±2.44^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.2±0.20^{a}	7.6±2.32^{ab}
	200.0	27.6±11.42^{a}	0.2±0.20^{a}	0.6±0.25^{a}	12.2±4.99^{b}	0.2±0.20^{a}	0.2±0.20^{a}	1.2±0.49^{b}	14.6±5.34^{b}
E. bicolor	0.0	103.8±4.63^{c}	0.0±0.00^{a}	0.2±0.20^{a}	0.2±0.20^{a}	0.0± 0.00^{a}	0.0± 0.00^{a}	0.2±0.20^{a}	0.6±0.25^{a}
	0.2	94.2±2.60^{b}	0.2±0.20^{a}	0.6±0.40^{a}	0.4±0.40^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.2±0.49^{ab}
	25.0	90.2±1.88^{b}	0.0±0.00^{a}	0.4±0.25^{a}	1.4±0.75^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.8±0.86^{ab}
	200.0	79.0±1.30^{a}	0.4±0.25^{a}	0.8±0.37^{a}	1.2±0.49^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.4±0.40^{a}	3.0±1.05^{b}

MN = Micronucleated cells; Values expressed as mean ± SD.

^{a,b,c} = Values in the same column, in each plant species, followed by the same letter are not significantly different ($P < 0.05$).

Table 3 Mitotic index, chromosome aberrations and mitotic aberration index of *Allium cepa* and *Eucrosia bicolor* root tip cells treated with various concentrations of $CuCl_2$ for 24 hr.

Plant species	Test concentration ($mg.L^{-1}$)	Mitotic index	Chromosome aberration (‰)					Mitotic aberration index
			Bridge	Disturbed	MN	Laggard	Sticky	
A. cepa	0.0	117.8±2.84^{c}	0.0±0.00^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.2±0.20^{a}
	1	111.6±3.49^{c}	0.0±0.00^{a}	0.2±0.20^{a}	0.8±0.37^{a}	0.2±0.20^{a}	0.2±0.20^{a}	1.4±0.25^{ab}
	2	94.0±1.18^{b}	0.4±0.25^{a}	0.8±0.80^{a}	1.6±0.87^{a}	0.4±0.25^{a}	0.0±0.00^{a}	3.2±0.97^{bc}
	3	80.4±2.34^{a}	0.4±0.25^{a}	12.0±0.49^{b}	1.6±1.12^{a}	0.0±0.00^{a}	1.0±0.45^{b}	4.2±1.24^{c}
E. bicolor	0.0	114.8±2.06^{c}	0.0±0.00^{a}	0.2±0.20^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.4±0.25^{a}
	1	104.8±2.54^{b}	0.4±0.40^{a}	0.4±0.25^{ab}	0.4±0.25^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.2±0.37^{a}
	2	85.0±2.05^{a}	0.0±0.00^{a}	0.8±0.37^{ab}	0.8±0.37^{a}	0.2±0.20^{a}	0.0±0.00^{a}	1.8±0.49^{ab}
	3	82.0±3.45^{a}	0.8±0.58^{a}	1.6±0.68^{b}	0.4±0.25^{a}	0.0±0.00^{a}	0.0±0.00^{a}	2.8±0.80^{b}

MN = Micronucleated cells; Values expressed as mean ± SD.

^{a,b,c} = Values in the same column, in each plant species, followed by the same letter are not significantly different ($P < 0.05$).

exposed to the lowest dose, as shown in Table 3. In contrast, *A. cepa* was judged to be copper-cytotoxic tolerant from the significant decrease in the ‰MI at only the higher tested doses (2 and 3 $mg.L^{-1}$). An increase in the Cu concentrations was found to be associated with an increase in the ‰MA in both plants. The highest frequency of chromosome aberration type induced by Cu was different from Pb, being disturbed mitotic aberration in the former and MN in the latter.

Slightly less sensitivity to As toxicity was shown in the *E. bicolor* root tip cells. Unlike *A. cepa*, the cytotoxic and genotoxic effect of As significantly reduced the ‰MI and increased the ‰MA in *E. bicolor* only at the highest dose of 10 $mg.L^{-1}$ $NaAsO_2$ (Table 4). The types of aberration induced by As were different between *E. bicolor* and *A. cepa*. In *E. bicolor*, two aberration types—namely, disturbed (1.20-1.40‰) and sticky (1.40‰)—were significantly induced by 1.00 and 10.00 $mg.L^{-1}$ of $NaAsO_2$ compared with the control. In the case of *A. cepa*, only the sticky type was significantly induced by As (Table 4).

DISCUSSION

This is the first report which has investigated and explored the potential use of *E. bicolor* as a heavy-metal cytotoxic and genotoxic bioindicator. Sensitivity to heavy metals was shown to be species dependent. In the case of Cd, only *E. bicolor* and *A. cepa* were significantly affected by a cadmium chloride solution in a dose dependent manner, while the responses to Cd fluctuated in the other three plant species. Sensitivity to Cd toxicity has been reported to be different among plant species (An, 2004; Unyayar *et al.*, 2006). An (2004) suggested that *Sorghum bicolor* was more sensitive to Cd toxicity than *Z. mays, Triticum aestivum* and *Cucumis sativas* in acute toxicity testing as indicated by the root growth endpoint. Cadmium nitrate ($CdNO_3$) induced higher percentages of micronucleate cells (%MN) in *Vicia faba* than *Allium sativum* and thus

Table 4 Mitotic index, chromosome aberrations and mitotic aberration index of *Allium cepa* and *Eucrosia bicolor* root tip cells treated with various concentrations of $NaAsO_2$ for 24 hr.

Plant species	Test concentration ($mg.L^{-1}$)	Mitotic index	Chromosome aberration (‰)					Mitotic aberration index
			Bridge	Disturbed	MN	Laggard	Sticky	
A. cepa	0.0	110.0±3.52^{c}	0.4±0.25^{ab}	0.0±0.00^{a}	0.6±0.40^{a}	0.2±0.20^{a}	0.0±0.00^{a}	1.2±0.58^{a}
	0.25	91.2±1.59^{b}	0.8±0.20^{b}	0.4±0.25^{a}	1.6±0.75^{a}	1.2±0.20^{b}	0.0±0.00^{a}	4.4±0.81^{b}
	1	95.8±1.99^{b}	0.4±0.25^{ab}	1.4±0.93^{a}	1.4±0.75^{a}	0.8±0.49^{ab}	0.0±0.00^{a}	4.4±1.21^{b}
	10	80.6±2.87^{a}	0.0±0.00^{a}	0.8±0.58^{b}	1.0±0.55^{a}	0.4±0.25^{ab}	2.2±0.97^{b}	4.8±0.49^{b}
E. bicolor	0.0	103.8±4.63^{b}	0.0±0.00^{a}	0.2±0.20^{a}	0.2±0.20^{a}	0.0±0.00^{a}	0.2±0.20^{a}	0.6±0.25^{a}
	0.25	92.2±2.82^{ab}	0.0±0.00^{a}	0.8±0.37^{ab}	0.2±0.20^{a}	0.2±0.20^{a}	0.0±0.00^{a}	1.2±0.20^{a}
	1	90.0±7.13^{ab}	0.0±0.00^{a}	1.4±0.25^{b}	0.2±0.20^{a}	0.0±0.00^{a}	0.0±0.00^{a}	1.6±0.40^{ab}
	10	80.4±3.27^{a}	0.0±0.00^{a}	1.2±0.37^{b}	0.2±0.20^{a}	0.0±0.00^{a}	1.4±0.51^{b}	2.8±0.80^{b}

MN = Micronucleated cells, Values expressed as mean ± SD.

^{a,b,c} = Values in the same column, in each plant species, followed by the same letter are not significantly different ($P < 0.05$).

V. faba could be considered as a Cd-sensitive plant (Unyayar *et al.*, 2006). Consequently, it can be assumed that sensitivity and tolerance to Cd are controlled by genetic factors, as Cd-tolerant plants appear to be able to prevent the excess absorption of Cd, and also to detoxify the Cd after it has penetrated plant cells (Das *et al.*,1997; Hocking and McLaughlin, 2000). Different mechanisms have been proposed to deal with the tolerance to heavy metal stress in plants, for instance, heavy metal immobilization, compartmentalization and synthesis of stress proteins (Li and Xiong, 2004).

In the present studies, the cytotoxicity and genotoxicity of Cd was investigated by observing the decrease in the ‰MI and the increase in the ‰MA in root tip cells. The current findings agreed well with other reports which showed cytotoxicity and also genotoxicity of Cd in plant cells (Unyayar *et al.*, 2006; Mishra *et al.*, 2007). Seth *et al.* (2008) suggested that exposure to Cd prevented cells entering cell division phases which then resulted in a decrease in the MI. Additionally, the primary action of Cd on the mitotic spindle promoted spindle-related abnormalities such as laggard chromosomes and bridges during cell division. The mitotic depression caused by heavy metals has been claimed to prevent a number of cells entering the prophase and thus blocking the mitotic phase of the cell cycle (Yildiz *et al.*, 2009). Considering the three sub-phases of interphase, there are a number of reports showing that either the synthetic phase (S) or the gap2 phase (G2) were frequently disturbed by cyto-genotoxic substances (Van't Hoff, 1968; Webster and Davidson, 1969; Salehzadeh *et al.*, 2003). According to Macleod (1969) and Brunori (1971), disturbances of the interphase stage at S or G2 could occur through inhibition of the sub-phases or an increase in the phase duration. Significant differences in mitotic cell division and mitotic aberration at the lowest investigated dose of Cd (0.02 $mg.L^{-1}$) in *E. bicolor* showed that the sensitivity to Cd cytotoxicity was similar to that found with *A. cepa*. Even though the results showed that *A. cepa* var. ascolonicum, *Z. rosea*, and *W. triloba*, had higher percentages of dividing cells (132.40, 86.20 and 83.0%, respectively) as shown in Figure 2, but they all had less sensitivity to Cd. Consequently, these plant species were omitted from further experiments.

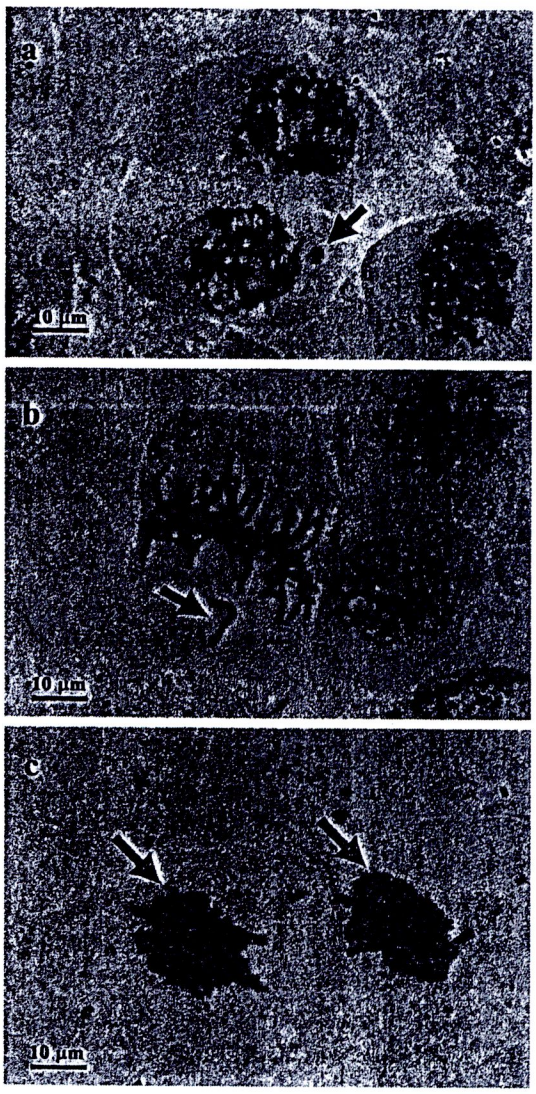

Figure 2 Aberration of interphase nucleus and mitotic cells in heavy-metal-treated root tips from *Eucrosia bicolor*. Abnormalities were observed (arrowheads) as: (a) micronucleus, (b) disturbed metaphase, and (c) nuclei sticky at telophase.

The results of the exposure to Cd for different periods of time showed consistent effects of cyto-genotoxicity measured by the two biomarkers namely, the decrease in the ‰MI and the increase in the ‰MA. Even though the higher indices of toxicity as depicted by the two biomarkers, would have to be subjected to a longer period of treatment, the shorter period of treatment still clearly showed cyto-genotoxicity caused by heavy metals (Table 1). In order to save time in testing, it was proposed that the shorter exposure time of 24 hr was sufficient and reliable. The current results also support the work of Das *et al.* (1997) who showed that cadmium could penetrate into plant cells and consequently induced genetic damage within an exposure time of just 24 hr.

The potential of *E. bicolor* as a cyto-genotoxic bioindicator for assessing other heavy metals contamination was investigated in the current study in experiments carried out in parallel with the *A. cepa* tests. The results showed that susceptibility to heavy metal toxicity was influenced by plant species as well as the concentration of the test chemicals. The response by *E. bicolor* to Pb toxicity was similar to that of *A. cepa* as represented by the decrease in the ‰MI and the increase in the ‰MA in the Pb-treated groups. A subsequent focus on the cytotoxicity of Pb which indicated an effect at the lowest dose, as was observed with *A. cepa*, implies that *E. bicolor* should be recognized equally with *A. cepa* as a Pb- sensitive plant. Furthermore, the results also showed that the cytotoxic endpoint (‰MI) recorded from the root tip cells of both plant species could be used as a biomarker for the assessment of Pb contamination at the permissible dose (0.2 $mg.L^{-1}$) of Pb contamination in waste water set by government (Thai Ministry of Science, Technology and Environment, 1996). Pb has been reported as an effective mitotic poison as well as a turbagen due to its ability to induce different types of spindle disturbance (Patra *et al.*, 2004; Kumar and Tripathi, 2008). Abnormality of mitotic spindle formation and function has been shown to be the cause of laggard chromosomes, disturbed and MN formation, with the latter two being found in the current investigation and representing the two highest frequencies of aberration type. Notably, MN was induced at high frequencies by $Pb(NO_3)_2$ in both plant species. It is most likely that the levels of lead nitrate toxicity are similar in these two plants.

Even though the results showed a greater sensitivity by *E. bicolor* to Cu toxicity than by *A. cepa* at the lowest investigated doses (1.00 $mg.L^{-1}$) of $CuCl_2$, the same type of aberration (mitotic disturbance) was induced in both plant species. The cytotoxicity and genotoxicity of Cu presented in this study were consistent with the report of Yildiz *et al.* (2009) who showed the effects of Cu on inducing chromosome aberration, DNA damage and root growth retardation in *A. cepa*. The results also support the classification of heavy metals with respect to the effect on cell division where copper showed marked effects like chromium (Cr), cobalt (Co) and Cd (Patra *et al.*, 2004). It is interesting to observe that the root tip cells of *E. bicolor* responded to $CuCl_2$ at concentrations as low as 1.00 $mg.L^{-1}$. This concentration is lower than the permissible level (2.00 $mg.L^{-1}$) of Cu in waste water (Thai Ministry of Science, Technology and Environment, 1996).

E. bicolor showed slightly less cytotoxicity and genotoxicity response to As than did *A. cepa*, based on the decrease in the ‰MI and the increase in the ‰MA which were significantly different from the control only at high concentrations of $NaAsO_2$ (up to 10.00 $mg.L^{-1}$). Yi *et al.* (2007) reported on the sensitivity of *A. cepa* to As at a concentration ranging from 0.3 to 100.0 $mg.L^{-1}$ as assessed by a micronucleus test. The researchers suggested that the decrease in the ‰MI might have been the result of a slower progression through the synthetic (S) phase during mitotic cell division. The effects of As on the spindle apparatus and chromosomal peripheral proteins were shown by disturbed and chromosome stickiness formation in both *A. cepa* and *E. bicolor* in the current study.

These two types of abnormality usually lead to chromosome bridges and breaks which implies the potential of arsenic as clastogenic (Souguir *et al.*, 2008).

Kwaunkua *et al.* (2010) reported on the use of *Eucrosia bicolor* as a genotoxic bioindicator. However, its high potential at a level comparable to *A. cepa* could not be determined at the time until a more thorough study was carried out as in this report. Even though *E. bicolor* is native to Peru and Ecuador, it can be found growing throughout Thailand Kwaunkua *et al.* (2010). This tropical bulb species is very easy to propagate and is generally used as a pot plant for landscape design. The general appearance of *E. bicolor*, as shown in Figure 3, suggests that the plant could thrive in a normal tropical environment. A clone of *E. bicolor* used in this research has the somatic chromosome number of 2n=50. Even though *E. bicolor* has a relatively high number of chromosomes, it was found that chromosome aberrations could be observed clearly due to the large size of the chromosomes in this plant species.

Figure 3 *Eucrosia bicolor*, grown in pots showing: (a) healthy plants; (b) flowers; (c) root bulbs; and (d) in use for assessing genotoxicity.

CONCLUSION

The degree of sensitivity to heavy metal cyto-genotoxicity in plants was shown to be species dependent. Of the five species tested, two (*E. bicolor* and *A. cepa*) were found to be Cd-sensitive plants. An exposure time of 24 hr was sufficient to produce a response that *E. bicolor* was a reliable cyto-genotoxic bioindicator for Cd toxicity. Testing with three other heavy metals—namely, Pb, Cu and As—showed that *E. bicolor* can be used as an alternative heavy-metal cyto-genotoxic bioindicator. This tropical bulb species was equally sensitive to Pb as *A. cepa*, but more sensitive than *A. cepa* in Cu genotoxicity testing.

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